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REVIEW ARTICLE

Use of Cellular and Cytokine Adjuvants in the Immunotherapy of Cancer

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Cellular and cytokine adjuvants, often immune effector cells and soluble factors, respectively, are supplemental and/or follow-up treatments of human origin for cancer patients who have unsatisfactory clinical responses to conventional chemotherapy, radiotherapy, and surgery. Since many human studies with these reagents are in their infancy, extensive data collection is only now being performed to determine which strategy provides the greatest therapeutic benefit. Research published in the literature since the genesis of this approach to cancer treatment is summarized in this report. Methodologies attempting to generate anticancer responses by provoking or enhancing the patient's own immune system are new compared with the other standard types of cancer treatment. Although a few encouraging human studies can be discussed, many of the most promising techniques are only now being transferred from the laboratory to the clinic. The administration of immune effector cells in combination with immunomodulators, such as interferons or interleukins, often enhances clinical outcome. The literature cited in this report indicate that immune-cell- and cytokine-based therapies hold promise in our attempts to improve the quality and duration of life in those with cancer. With each report reaching the literature, more effective clinical trials are being designed and implemented. *J. Surg. Oncol. 1998;68:122–138.*

KEY WORDS: immunotherapy; adjuvant; neoplasms; dendritic cells; cytokines; lymphocytes

INTRODUCTION

Since the mechanism of the molecular events involved in immune recognition has been elucidated, new and exciting strategies in anticancer therapeutics have opened. Researchers now understand some of the crucial portions of primary and secondary signaling pathways that are activated when T and B lymphocytes produce an immune response to a tumor cell (reviewed in references 1–5). Mature B cells, the principle effectors of the humoral arm of the immune response, develop into antibody-secreting, activated B lymphocytes following stimulation by antigen in its native form. T cell (cell-mediated) recognition of antigen requires the formation of a trimolecular complex comprised of: (1) the major histocompatibility complex (MHC), (2) the T cell receptor (TCR), and (3) a short segment of intracellularly processed protein associated with the MHC. Antigen presentation of cell-surface peptides to T cells can occur in association with either MHC class I or II molecules: the former associated with $CD8^+$ T cell responses (usually cytolytic/cytotoxic T cells, or CTL), and the latter associated with $CD4^+$ T cell responses (usually helper T cells, or T_H). Two distinct pathways exist for loading of antigenic peptides into MHC molecules; one which involves class I MHC and

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the other associated with class II MHC. The major route for presentation on antigens on class I molecules involves translocation of peptides from the cytoplasm into the endoplasmic reticulum where peptides can bind to newly synthesized class I molecules. The peptide binding site of MHC class I molecules can accept peptides of 8 to 10 amino acids in length which contain consensus binding motifs. The binding motif for each class I allele is different, resulting in presentation of a different spectrum of peptides. Binding of peptide to class I stabilizes the class I-β2 microglobulin complex and allows it to be transported to the surface. Since peptides must be in the cytoplasm to be loaded into class I molecules, most peptides bound to class I are derived from intracellular proteins. These intracellular proteins include proteins made by intracellular parasites such as viruses and tumor-associated antigens (TAA). Because proteins produced endogenously within tumor cells are likely to be presented on class I molecules, and since class I MHC-peptide complexes are recognized by $CD8^+$ T cells, stimulation primarily of TAA specific $CD8^+$ T cells is the goal of antitumor vaccines.

The pathway which loads antigenic peptides onto class II MHC molecules captures a distinct set of proteins. Following synthesis, class II molecules are tightly bound to invariant chain protein and are found in specialized vesicles. These vesicles fuse with lysosomes containing proteins phagocytosed from the extracellular environment. In this low-pH environment, removal of the invariant chain from class II is facilitated by the HLA-DM protein, allowing binding of antigenic peptides. The newly formed class II-peptide complexes are then transported to the cell surface. Therefore, class II MHC molecules bind peptide antigens predominantly derived from outside the cell such as bacterial proteins, toxins, and extracellular parasites.

Significant progress has been made in the discovery and characterization of TAA, beginning with the identification of MAGE earlier in this decade [6,7]. This has led to intensive research into these moieties as potential targets of immune-based cancer treatment. These targets can be classified into four general groups, and are summarized in Table I. The first group contains what is commonly referred to as the ''cancer/testis'' antigens, whose expression in normal tissues is limited to testis and whose genes have been mapped to the X chromosome. The MAGE (Melanoma AntiGEn) gene family was initially discovered by stably transfecting melanoma cell line DNA into cells expressing the appropriate MHC molecule and then screening for the gene-conferring reactivity to tumor-specific T lymphocytes [6]. Subsequently it has been shown that the expression of this gene family in normal tissues is limited to spermatogonia. Since these cells do not express MHC (required for the presentation of tumor antigens to the immune system), many researchers consider the expression of these genes to be tumor-specific. A second group consists of virally derived antigens, of which the association of the E6 and E7 proteins from certain Human Papilloma Virus (HPV) strains with cervical cancer has a particularly strong correlation [8]. A third group, differentiation antigens, are those expressed in both normal and neoplastic cells of the same tissue, albeit usually at enhanced levels in the latter [9]. Since these are self-antigens, their use as an immunotherapy target likely necessitates that immune tolerance be overcome or abolished. Lastly, the DNA or protein sequences of several tumor antigens are in a modified or mutated state in tumor as compared to normal tissue [10–14]. A member of this group, the mucin MUC-1, is hypoglycosylated and nonpolarized on tumors, exposing epitopes capable of stimulating cytotoxic T cells [15]. From this harvest of potential targets has arisen numerous clinical trials, in various stages of completion, that attempt to enhance the host's immune response to tumor. There are difficulties with the general approach, including selection for tumor-escape variants and the potential of breaking tolerance to self-antigens. However, a comprehensive discussion of the limitations of immunotherapy is beyond the scope of this article.

This review concentrates on some of the more promising cancer therapies that are based on the premise that the human body can be stimulated to mount an immune response sufficient to improve survival and quality of life. We attempted to focus primarily on human studies, but where the technology is too new for extensive clinical information, we cited preclinical and animal research. In other areas in which a body of clinical data exists, such as tumor-infiltrating lymphocyte- (TIL)-based adoptive transfer, the studies we mention may only hint at the amount of information available and its potential promise. In order to limit the vast scope of immunotherapeutic studies covered, we did not address many exciting immunotherapeutic methodologies, including peptide-based active immunization, monoclonal antibodies, immunomodulating anticancer chemotherapy, carrier-based conjugates (e.g., QS-21, BCG, and KLH), and others [16–23]. In addition, we avoided comparisons between immunotherapy and early indications of clinical benefit using recently developed, promising chemotherapy agents such as Topotecan® [24], paclitaxel [25], Taxotere® [26], and gemcitabine [27]. Especially for those familiar with such more-established modalities (including radiotherapy and surgery), we hope this review provides an introduction to a few aspects of what is one of the most promising approaches to cancer treatment in several decades.

ANTIGEN-PRESENTING CELLS (APC)

Specific immune reactivity to tumor antigens produced intracellularly does not arise from direct interac-

TABLE I. Human Tumor Antigens Recognized by T Lymphocytes

Antigen	Tumor	Reference
X chromosome-associated ''cancer-testis'' antigens		
MAGE-1 and -3	Melanoma, nonsmall cell lung, head and neck, bladder, breast sarcoma, prostate, colon	[6,9,161]
BAGE	Melanoma, nonsmall cell lung, head and neck, bladder, breast sarcoma	[162,163]
GAGE-1	Melanoma, nonsmall cell lung, ovarian	[163–165]
RAGE	Renal cell, sarcoma	[163,166]
HOM-MEL-40	Melanoma	[167]
NY-ESO-1	Melanoma	[168]
GnT-V	Melanoma	[169]
Virally-derived antigens		
HPV	Cervical	[170]
EBV	Nasopharyngeal, Hodgkin lymphoma	[171,172]
Differentiation antigens		
PSA	Prostate	[173]
PSMA	Prostate	[174–176]
MART-1/Melan-A	Melanoma	[177,178]
Tyrosinase	Melanoma	[179,180]
gp100/Pmel17	Melanoma	[181–183]
TRP-1/gp75	Melanoma	[184]
p15	Melanoma	[185]
Ganglioside GM_2	Melanoma	[186]
Antigens modified or mutated in tumor tissue		
β-catenin	Melanoma	[10]
ras	Pancreatic, colon, lung, urinary tract, bladder, others	[11,12,187]
p53	Colon, breast, lung, renal, others	[13,14]
CDK4	Melanoma	[188]
$p185^{HER\text{-}2/neu}$	Ovarian, breast	[189–192]
MUC-1	Breast	[15,193]

tion of T lymphocyte with intact protein. This was first demonstrated by showing that influenza-specific CTL could recognize proteins that were not part of the membrane of infected cells [1]. Building upon work such work with viral antigens, it was shown that tumor cell proteins were processed intracellularly into short peptide fragments before being presented on the cell surface by MHC molecules [28]. Tumor cells may not possess the necessary costimulatory molecules for immune recognition and response. Thus, it was theorized that cells capable of presenting antigen (APC) and having the requisite costimulatory capacity greatly augment antitumor responses. All cells that express MHC proteins are, to some extent, APC; they can utilize such proteins for binding and presentation of antigen to cognate receptors on T cells. What distinguishes three major groups of APC from the others is their unique ability to acquire and retain antigen, their capacity to stimulate antigen-restricted clones, and the production of systemic immunity [29]. The three major groups of APC identified as having such characteristics are: (1) dendritic cells, (2) cells of the monocyte/macrophage lineage, and (3) B cells. The third type of APC, the B cell, is primarily a soluble antigen-presenting accessory cell to T_H populations, usually via immunoglobulin surface receptors. B cells efficiently take up antigen for presentation to helper T cells rather than cytotoxic T cells; they abundantly express MHC class II but are class I negative [30]. Since this review concerns cell-mediated (MHC class I) rather than humoral (MHC class II) antitumor immunity, B cells are not mentioned further.

DENDRITIC CELLS (DC)

Progenitor DC are leukocytes originating in the bone marrow. They are considered immature following migration to nonlymphoid tissues such as the epidermal layer of the skin, the respiratory and gastrointestinal systems, and interstitial regions of several solid organs [31]. In a recent report using an in vivo rat model, Matsuno et al. [32] indicated that bone marrow-derived dendritic cells

travel to the Kupffer cells of the liver, where they demonstrate transient phagocytic activity. Subsequently, endocytic function appears to be downregulated; once DC have taken up antigen, they subsequently translocate to the lymph, where they no longer present markers indicative of such function. While still immature—and even after maturation—DC possess unique capabilities for antigen recognition, uptake, and processing [33–35]. They are a vital accessory cell for the stimulation of both $CD8^+$ and $CD4^+$ T cell subsets [36]. In vivo studies indicate that DC furnish all the needed signals to stimulate naive T cells, as a result of trimolecular complex formation, as well as the binding of costimulatory molecules on the DC to receptors on the T cell surface [37]. Potent T cell reactivity could be provoked using very low DC:T cell ratios (1:50–1:200), although there is contradictory data as to whether or not accessory $CD4^+$ T_H cells are required [38,39]. The usually nominal ability of resting B cells to process and present antigen to T cells was greatly enhanced following antigen-specific interaction with DC, perhaps due to upregulation of MHC class II and B7/BB1; the latter demonstrating increased binding to CD28 [40]. A high level of macropinocytosis was constitutive, and such antigen capture by DC was facilitated by the mannose receptor-mediated uptake and concentration into macromolecules associated with the MHC class II compartment [41]. The study of DC-based immunotherapeutics was facilitated by the improvement of isolation methods. DC could be collected using CD34+ hematopoietic progenitor cells from human umbilical cords or simply from peripheral blood [42–46]. Depending on the methodology, various cytokines were utilized for propagation, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4), and tumor necrosis factor (TNF)-α.

Due to their relatively recent use in experimental immunotherapies directed against human cancers, most of the data indicating the potential of DC-based treatments comes from animal models. At first, it was demonstrated that freshly isolated splenic-derived DC that were pulsed with sperm whale myoglobulin could stimulate antigen-restricted T cell responses in naive mice. The induction of functional T lymphoblasts required only $1–3 \times 10^5$ DC, and was limited to the parental strain of the DC used in priming [47]. T cells responded to fewer than 2,000 molecules of the superantigen staphylococcal enterotoxin A (a superantigen being defined as a bacterial or viral protein capable of binding to MHC class II as unprocessed product and activating large numbers of T lymphocytes [48]) if presented by DCs, due in large part to the capacity of DCs to bind 30–200 times more staphylococcal enterotoxin A than monocytes or B cells [49]. Dendritic cells were also a superior antigen delivery vehicle as compared with cells of macrophage/monocyte lineage; for example, only DC could elicit CTL specific for the glycoprotein B of herpes simplex virus type 1 (HSV-1) [50]. Further, in the absence of adjuvant, antigen-specific CD8+, CTL could sometimes be elicited following vaccination only when peptide-loaded DC were used as antigen-presenting cells (APC) [51,52]. Murine DC, lipofected with RNA from ovalbumin (OVA), were much stronger inducers of primary CTL reactivity as compared with similarly treated monocytes [53].

DC have been gene-modified in an effort to enhance their utility in tumor vaccine strategies. Alijagic and associates [54] were the first to report the transfection of DC with TAA: tyrosinase, a crucial enzyme in melanocyte development. A full-length tyrosinase cDNA was cloned into the pCEP4 mammalian expression vector, and the construct was subsequently lipofected into DC. Using reverse transcriptase (RT)-PCR and a tyrosinase hydroxylase assay, researchers were able detect gene expression at the mRNA and protein levels [54]. In another instance, MART-1 (melanoma antigen recognized by T cells) expression in DC could be demonstrated by use of a retroviral construct in which the gene of interest was produced under the control of the LTR from Moloney murine leukemia virus. MART-1-transduced DC elicited antigen-restricted CTL that secreted significant levels of IFN-γ following co-cultivation with HLA-A2+, MART-1+-established cell lines, and pulsed targets [55]. Gene expression using retroviral vectors was also used to drive protein production of MUC-1 in transduced DC [56] and to generate CD8+ lymphocytes in vitro specific for the gene of interest.

In vivo protection and active treatment research in the murine system has confirmed that DC could be efficacious in the enhancement of human antitumor immunity. In one experiment, human papilloma virus (HPV) 16 E7(49–57) peptide was loaded on DC and used as an immunogen against outgrowth of HPV 16-transformed B6 mouse embryo (C3) cells. The observation that three-fourths of the mice given DC-peptide treatment remained tumor free 60 days after challenge was due to the APC, since the percentage of tumor-free animals receiving free peptide was not significantly different from naive mice [52]. Bone marrow-derived murine DC were pulsed with OVA peptide, then used in a protection experiment against challenge with MO5, an OVA-transfected B16 tumor cell line. Eighty percent of treated mice survived for more than 7 months following the injection of a lethal dose of MO5, while all naive mice died by day 21 [57]. This type of protective immunity using peptide-pulsed DC was shown to be antigen-specific, largely $CD8^+$- and $CD4^+$-dependent, and could be blocked by administration of monoclonal antibodies (MAb) against cytokines that mediate T-helper cell 1 (T_H1) activity, such as IFN-γ, TNF-α, and IL-12 [58]. Genetic immunizations using the ''gene gun'' methodology have been used to treat tumors in a murine model, allowing for controlled intro-

duction of DNA into DC in the absence of viral vectors [59]. OVA DNA was coated onto gold particles and cutaneously shot into the shaved abdominal area of C57BL/6 mice. Splenocytes from immunized animals exhibited cytolytic activity against the OVA-transfected lymphoma, EG7, but not the untransfected parental cell line, EL4. Antigen-restricted, CD8+-mediated protection was shown in protection experiments by the ability of genetically vaccinated mice to survive a lethal challenge of MO4, a similar OVA-transfected B16 tumor cell line to the one previously described. Using plasmid DNA encoding the Lantern variant of the reporter gene for green fluorescent protein (GFP), investigators were able to demonstrate selective expression of GFP in DC obtained from the draining lymph nodes of GFP-immunized mice. Using fluorescence microscopy, it was demonstrated that the DC likely originated from immunized skin, and that active production of GFP was accomplished [59]. Thus, biolistic immunization to cutaneous DC was shown to be yet another potential strategy to elicit potent, antigen-restricted, protective tumor immunity.

In another study, soluble β-gal protein was cocultivated with DC, allowing for antigen uptake, processing, and presentation. Ten to 12 days following vaccination with 5×10^5 DC, mice were injected with F1 tumor cells, a spontaneously transformed BALB/c fibroblast line transduced with a β-gal-expressing retrovirus. Nearly two-thirds of challenged animals were protected from tumor growth, and tumor growth was abolished in mice receiving an additional boost with soluble β-gal protein [60]. In a similar report, protective immunity against the IgM-expressing lymphoma cell line, BCL1, was elicited using DC pulsed in vivo with soluble IgM [61]. Boczkowski and coworkers [53] used DC transfected with total, poly A+, and in vitro transcribed RNA from OVA to evoke antitumor immunity in mice immunized with murine thymoma cells expressing OVA. Tumor growth was significantly abrogated in mice given DC transfected with OVA RNA at the time of tumor challenge. In an active treatment model, mice bearing established tumor were vaccinated three times with tumor RNA-transfected DC. Compared with controls, significant regression of lung metastases was observed [53]. The advantage of these approaches was that T cell reactivity could be generated in the absence of a well-defined peptide target.

In clinical trials, data is beginning to be collected which indicates that DC can provoke a tumor-specific immune response to TAA. This has been demonstrated even when such TAA are self-peptides, and thus the recipient is theoretically tolerized. In a recent experimental human trial with B cell lymphoma, Hsu et al. [62] reported on four patients given DC-based vaccinations. DC were isolated following leukapheresis, then co-cultured for 24 hr with idiotypic immunoglobulin derived from the autologous lymphoma. Intravenous administration of $2.0–32 \times 10^6$ occurred on day 2, followed by a subcutaneous administration of idiotype (Id) protein on day 16. Three additional vaccinations with DC followed by idiotype protein were given on days 28 and 56, as well as 5–6 months after the initiation of treatment. No significant side effects were observed. One complete and one partial response were noted, and a third patient, while having ambiguous CT scans, had no evidence of tumor in bone marrow (BM) and peripheral blood mononuclear cells (PBMC) by PCR analysis. As measured by thymidine incorporation assay, CTL from all patients exhibited significant cell proliferative reactivity following in vitro culturing with Id protein after 1–3 vaccinations. CTL from one patient was cytolytic toward its autologous tumor hybridoma after 5 weeks of in vitro stimulation [62]. Although this strategy requires that idiotypic protein be produced for each recipient, it provides some of the strongest evidence that antigen-pulsed DC represent an effective method to treat established human cancer by provoking the host's immune system.

Further indication of the potential of DC-based immunotherapeutics come from recent phase I clinical trials for prostate cancer [63] and melanoma [64]. Murphy et al. [63] treated 51 hormone-refractory men with infusions of autologous DC, DC plus either of two PSMA-derived peptides, or with each peptide alone. Forty-seven (92%) of patients were stage D0–D2, and all had elevated PSA serum levels. PSMA peptides with favorable amino acid anchor residues in accordance with the HLA-A0201 motif [65] (PSM-P1: LLHETDSAV; PSM-P2: ALFDIESKV) were chosen as immunogens. Whole PSMA had previously been shown to be effective in evoking strong in vitro cellular reactivity in prostate cancer patients [66,67]. Within the three treatment groups given DC, patients were randomly selected to receive a dose of 1, 5, 10, or 20×10^6 cells. Regardless of dose, the vaccinations were well tolerated; occasional and transient hypotension was the only side effect observed. The advanced age and poor health of the patients resulted in meager PBMC collection for immune monitoring beyond that dedicated to the generation of DC. However, lymphoproliferative responses were observed in several patients following at least two infusions of DC or DC pulsed with PSM-P1 or -P2. Seven partial responses were identified, with the response lasting 150–225 days at the time of publication. Serum analysis showed significant decreases in total PSA, PSMA, and total alkaline phosphatase in 5 of 7 responders [63]. A more comprehensive phase II study is currently underway.

A clinical trial against melanoma is being carried out using DC exogenously pulsed with acid-stripped peptides derived from the melanocyte-lineage proteins MART-1 and gp100. Following in vitro expansion, four

injections of from 5–10 × 10^6 DC are being infused [64]. It is hoped that such studies will serve to further the promise that autologous DC as the basis of peptide-based or gene-modified immunotherapeutic approach, is more effective and less harmful than chemo- or radiotherapy.

MONOCYTES AND MACROPHAGES

Other cell populations derived from myeloid precursors, monocytes, and macrophages, have also served as APC in immunotherapeutic clinical trials. In the bone marrow, blood monocytes arise from promonocytes, after which they migrate in the tissues as mature macrophages. Like DC, these cells of the mononuclear phagocyte system possess the ability to phagocytize protein, then process it intracellularly and present it on the cell surface to immune effector and memory cells. Such cells are vital for immune recognition of antigen that has drained into lymphoid tissue from the site of introduction. Components of foreign invaders (such as bacterial endotoxins or cell components) or cytokines (macrophage activating factors, including IFN-γ) serve as activating agents [68]. Subsequently, macrophages and monocytes secrete several cytokines involved in immune response, including: (1) IL-6, which promotes growth and differentiation of T and B cells, (2) TNF-α, which enhances cytocidal activity towards tumor cells, and (3) IL-10, which helps mediate the overall immune response by inhibiting IFN-γ secretion. It appears that direct cell-cell contact initiates monocyte-tumor cell interaction, followed by vacuolation in the target cell, and eventual lysis [68].

As with other approaches to immune system-based cancer therapeutics, preliminary indications of the potential efficacy of macrophages/monocytes in cancer treatment arose from animal studies. In one of the earliest reports, these effector cell types were harvested from the peritoneal exudate (PE) of mice treated with bisantrene, an anthracene derivative used in the treatment of metastatic breast cancer [69]. Macrophages from mice given bisantrene demonstrated dose-dependent cytostasis of P815 mastocytoma cells. The abrogation of P815 proliferative capacity was potentiated by macrophages collected 2 days to 4 weeks following treatment, indicating long-term upregulation of macrophage function [70]. Peritoneal exudate cells obtained from bisantrene-stimulated animals were admixed with EL-4 lymphoma cells and administered intraperitoneally (i.p.) into B6 mice. One injection of PE cells could significantly enhance survival in this active treatment model. As compared with controls, mice given PE therapy demonstrated a 33% curative rate when challenged with a lethal dose of tumor cells. Since EL-4 cells were resistant to natural killer (NK)-mediated lysis, and since donor animals had not been exposed previously to EL-4 tumor antigens, the observed therapeutic benefit was not likely due to NK or T cells [71].

The feasibility of macrophage-based adoptive immunotherapy was demonstrated by a report by Andreesen and colleagues [72]. Fifteen patients with a variety of neoplasms including ovarian carcinoma, melanoma, and nonsmall cell lung cancer, were infused with autologous macrophages. Following leukapheresis, macrophages were expanded ex vivo in IFN-γ and subsequently separated from other mononuclear cells by density centrifugation. Patients received between 1–9 cycles of therapy at biweekly intervals. Mild fever, malaise, and fatigue were the only clinical side effects, which did not correlate with dosage (1.0–17.0 × 10^8 cells). No objective clinical responses were observed, although three patients demonstrated short-term stability of disease. However, elevated titers of serum neopterin and IL-6 in blood samples seemed to indicate a biologic response in a majority of patients [72]. A similar study of 11 patients with nonsmall cell lung cancer also showed that the adoptive transfer of macrophages activated ex vivo with IFN-γ could be well tolerated, but again this approach showed no therapeutic benefit and only a minimal biologic response [73]. In contrast to the aforementioned report [72], counterflow centrifugation-cell elutriation for monocyte/macrophage enrichment was used prior to ex vivo activation [73]. Although elutriated monocytes represented nearly 90% of the total cell population, more limited expansion in culture resulted in lower infusion dosages (0.1–5 × 10^8), albeit with fewer contaminating platelets or mononuclear cells than that described by Andreesen et al. [72]. No clinical responses were reported, although radioimaging studies with ^{111}In-labeled autologous macrophages showed initial trafficking to lungs, liver, and spleen. Mild to moderate chills and fever were the only significant side effects. Significant increases in serum neopterin were not observed until after six doses, and serum IL-6 was not elevated in any patient [73].

Minor clinical responses were obtained in a more recent phase I/II clinical trial aimed at the treatment of advanced colorectal cancer [74]. A large amount of mononuclear cells were collected by serial aphereses. Following gradient separation, cells were cultured for 7 days, with IFN-γ added during the final 18 hr in vitro for activation. Elutriation occurred 4 hr prior to reinfusion, and resulted in an average of 87% mononuclear cells being administered. Between 0.05–3.84 × 10^9 autologous-activated macrophages were given once per week for a total of six treatments. Once again, treatment was well tolerated, with only a low-grade fever detected in 28% of patients. No other toxicity was noted, and liver and renal function remained within normal parameters. Compared with pretreatment material, monocytes collected following reinfusion displayed enhanced, though highly variable, cytostatic and cytolytic activity against

the U937 established tumor cell line. However, despite reinfusing as much as 10 times the number of cells as compared to earlier trials, overt clinical responses were not seen in any of the 14 patients evaluated. Three patients demonstrated short-term stable disease with progression-free survival of 12–14 weeks [74].

In earlier work with animal models, many experimental murine tumors did not secrete sufficient cytokines to directly or indirectly (via T cells) activate or mobilize macrophages. Consequently, this led to inadequate recruitment of macrophages at the tumor site and ineffective antitumor responses [68]. Liposomes were shown to be effective drug carriers for the delivery of IFN-γ and other biological response modifiers to phagocytic cells in vivo, overcoming the problem of inadequate activation [68]. Faradji and associates [75] used liposomes containing muramyltripeptide (L-MTP) as a diffusible immunomodulator to enhance tumoricidal activity in vivo in a phase I clinical trial of patients with peritoneal carcinomatosis. The development of counterflow centrifugal elutriation had largely overcome the technical barrier of obtaining the necessary titers of monocytes for adoptive transfer in humans. Blood monocytes purified by counterflow elutriation were ex vivo-activated with L-MTP, then infused intraperitoneally (i.p.) once a week for five total treatments. Although no major clinical responses were reported, the treatment was well tolerated, with fever and chills the most common and severe side effect. Radioimaging studies with ^{111}In-labeled, activated monocytes demonstrated strong presence in the abdominal cavity, with little biodistribution from this region 7 days following infusion. Of great importance, monocyte activation was demonstrated by the elevated secretion of IL-1, IL-6, and TNF in the supernatants of monocytes cultured from patient material [75].

The limited number of reports suggest that monocyte-derived, activated macrophages are superior to fresh, blood monocytes in the ability to lyse tumor, respond to IFN-γ upregulation, and secrete greater levels of cytotoxic cytokines such as TNF-α. However, the therapeutic benefit of a macrophage-based approach to the treatment of cancer, especially if bulky tumor exists, is unclear at this juncture.

INTERLEUKIN-2 (IL-2) AND LYMPHOKINE-ACTIVATED KILLER CELLS (LAK)

IL-2 is a autocrine/paracrine T cell growth factor, produced primarily by $CD4^+$ T-helper lymphocytes. IL-2 augments the proliferation of activated CTL, TH cells, and, to a lesser extent, NK cells. IL-2 also enhances the secretion of cytokines such as IFN-γ and TNF-α [76]. Initial in vivo murine tumor studies had demonstrated that IL-2 could inhibit the development of 3-day liver and lung micrometastases in a dose-dependent manner [77]. A phase I clinical trial with IL-2 therapy alone was disappointing, as none of 66 metastatic melanoma patients responded to treatment [78]. A balance of escalated dose of IL-2 with minimal treatment-related mortality (>1.5%) and acceptable toxicity resulted in a 15% combined complete response (CR) + partial response (PR) against various cancers including melanoma, renal, colorectal, and breast cancers, and non-Hodgkin lymphoma [79]. More recently, 29 patients with stage IV colorectal cancer were given 15 doses of IL-2 and IFN-α with only four partial responses (17%) obtained [80]. Even less therapeutic benefit resulted from bolus IL-2 administration combined with the adoptive transfer of anti-CD3-stimulated T-killer cells [81]. In addition, capillary leak syndrome was noted in several of these studies.

However, the enhanced therapeutic benefit of combining IL-2 with LAK cells, usually harvested from peripheral blood and activated in vitro, was soon demonstrated in animal models [82,83] and rapidly progressed to human studies. A clinical trial of 180 patients treated with IL-2 and LAK cells resulted in a CR + PR rate of 35% and 21% for renal cell carcinoma and melanoma, respectively [84,85]. Duration of response varied from 1 month to over 5 years. Increased survival with this combined regimen was not observed in patients with kidney cancer. Histologic analysis of subcutaneous melanoma lesions revealed widespread infiltration of lymphocytes, suggesting that the mechanism of action was enhancement of local immune reactivity [77]. In one of the more encouraging studies, six melanoma patients who had failed previous treatment with chemotherapy and isolated perfusion were treated with $7–16 \times 10^9$ LAK cells plus bolus IL-2. All six responded to some extent, with 4 PR, 1 CR, and one patient with stable disease [86]. Blay et al. [87] administered a continuous infusion of IL-2 ($20–28 \times 10^6$ units/m^2/course) in combination with LAK cells ($4.8–7.5 \times 10^{10}$) to 25 patients with metastatic renal cell tumors, and obtained a 20% overall response rate. Interestingly, immunoradiometric analysis revealed that responders had significantly higher titers of TNF-α in sera collected 8 days following therapy as compared with nonresponders [87]. This may have been due to shared receptors between LAK-derived lymphotoxin and TNF-α. A larger, subsequent phase I/II study treated 68 evaluable metastatic renal cell cancer patients with a combination of IL-2, LAK, and IFN-α [88]. Four (24%; 3 CR, 1 PR) and 19 (37%; 6 CR, 13 PR) responders were obtained in the phase I and II protocols, respectively. Since no subgroup was given only IL-2 and LAK, it was not possible to determine whether or not the administration of IFN-α accounted for the improved therapeutic benefit as compared with the earlier report [87]. Higher TNF-α serum concentrations were obtained post- versus pretreatment, although any correlation with therapeutic benefit was not reported [88]. Thus, while IL-2 in combination with

IFN-α elicited higher serum TNF-α titers than IL-2 alone, it remained unclear if TNF-α induction is strongly associated with clinical outcome.

It remains debatable whether or not a clear benefit has been demonstrated of adoptive transfer of LAK over high-dose IL-2 alone, since most of the aforementioned studies examined the synergistic effects of the two compounds. And although it is now known that LAK cells originate from NK cells exposed to high doses of IL-2, the mechanism of LAK-mediated antitumorgenicity is still unclear. Since LAK cells are $CD3^-$, cytocidal activity is independent of the T cell receptor. In vitro studies indicate that direct binding, rather than cytokine factors like TNF-α largely determines the capacity of LAK to lyse tumor. The molecules involved in cell-cell adhesion are yet to be identified, since the ''classical'' adhesion factors (LFA-1, LFA-3, and ICAM-1) do not appear to be involved [89].

IL-2 AND TUMOR-INFILTRATING LYMPHOCYTES (TIL)

TIL were first isolated as cellular infiltrates of tumor nodules more than a decade ago. IL-2 receptors on the surface of many TIL allowed in vitro expansion by culturing in the presence of IL-2 [77]. After growth in the presence of IL-2 and autologous tumor, TIL demonstrated cytolytic activity against both autologous and HLA-matched allogeneic tumors of murine as well as human origin [90–93]. Although freshly harvested TIL contained both $CD8^+$ and $CD4^+$ T cells, an increasing predominance of $Thy\text{-}1^+/CD8^+/CD4^-$ and $TCR\alpha\beta^+/\gamma\delta^-$ cells was observed in long-term, lytic cultures. Also, Vβ expression became more restricted during the course of culturing, with long-term TIL populations often expressing a single dominant Vβ [94]. Infused TIL displayed long-term survival in murine models. In metastatic melanoma patients, infused indium-111-labeled TIL were shown to migrate and localize to tumor sites as rapidly as 24 hr following injection. Three to 40 times more radioactivity was detected in metastatic deposits as compared with normal tissue [95]. Besides being potent antitumor cells capable of efficient trafficking to tumor, TIL from distinct anatomic sites may display similar biologic activity against unique tumor-associated antigens [96], presumably making any cancerous regions potential sources of antitumor lymphocytes in a patient with metastatic disease.

In perhaps the most comprehensive study on the therapeutic benefit of IL-2/TIL immunotherapy, Rosenberg et al. [97] reported on a 5-year experience with 86 metastatic melanoma patients treated with autologous TIL and high-dose bolus IL-2 (720,000 IU/kg at 8-hr intervals). At least 10^{10} were administered, based on murine studies and the inability to obtain a 50% reduction in tumor burden when fewer TIL were given [92]. Thirty-four percent of patients displayed either a partial or complete clinical response, which compared favorably with the 17% overall response rate observed in melanoma or renal cell patients receiving only high-dose IL-2 [85]. Of interest, analysis of TIL cultures revealed various characteristics that were directly correlated with response. Among these were significant associations between therapeutic benefit and doubling time, site of origin, and duration in culture. Improved clinical responses were seen when TIL exhibited a shorter doubling time, or were derived from subcutaneous tumor rather than involved lymph nodes, or were cultured a shorter period of time [97].

A combined regimen of TIL, IL-2, and IFN-α was used to treat 11 patients with metastatic renal cell carcinoma [98]. IFN-α alone was administered prior to radical nephrectomy, as well as in conjunction with IL-2 (6×10^6 U/m^2/day IFN-α, 2×10^6 U/m^2/day IL-2) during the weeks of TIL infusion. $0.14–16 \times 10^{10}$ TIL were infused, consisting of >80% $CD3^+$ T cells and an average ratio of 2:1 $CD8^+$:$CD4^+$ lymphocytes. The treatment was not well tolerated: Reversible thyroid dysfunction, mild to moderate fever, chills, fatigue, diarrhea, and nausea occurred in at least 9/10 evaluable patients. In this and in another identical study, a 32% total response rate was observed (7 CR, 3 PR), with disease-free survival lasting over 3½ years [98]. This success rate was somewhat greater than previous protocols in which IL-2 and IFN were given without adjuvant TIL.

Several studies in recent years have attempted to explain the often disappointing results obtained with TIL-based immunotherapies. Gervois and associates [99] observed that the inefficient proliferation and IL-2 secretion of TIL following exposure to melanoma cells could be explained by expression of the appropriate MHC-peptide complexes at suboptimal levels. This suggested that the level of antigens expressed on tumors was a critical determinant of TIL activation. There is also mounting evidence that the TIL harvested from the patient, expanded ex vivo, and used for adoptive transfer could have profound intracellular abnormalities relating to antigen processing and presentation. In patients with colorectal carcinoma, renal cell carcinoma, and melanoma, T cells recovered from the tumor site expressed significantly decreased levels of the signal-transducing molecule CD3 ζ [100–103]. This defect might have clinical relevancy; it was observed in two studies that the overall survival of melanoma patients with low expression of signal transduction molecules such as CD3 ζ, $p56^{lck}$, and Zap-70 was significantly diminished compared with patients whose T cells exhibited normal expression [103]. Moreover, the abnormally low expression levels of CD3, $p56^{lck}$, and Zap-70 were not reversed following IL-2-based immunotherapy [100].

GENE TRANSDUCED TUMOR CELLS

Tumors are thought to escape immune recognition in part because they lack the ability to directly stimulate T cells [104–108]. A variety of genes have been transduced into tumor cells in attempts to enhance their antigen-presenting capabilities and, subsequently, their immunogenicity. Studies have focused on cytokine genes, costimulatory molecules such as B7, or alloantigens. The next generation of genetically modified tumor cell vaccines, which use combinations of cytokines or costimulatory molecules, is now being evaluated in animal models. This section will summarize representative work.

Expression of cytokine by tumor cells offers several advantages over systemic administration. Delivery of cytokines by intravenous or subcutaneous injection can be inefficient, especially when one considers the normal function of these molecules. In the course of a natural immune response, cytokines are produced at the site of immune cell sensitization. Within this local milieu, cytokines act in concert to activate immune cells, stimulate proliferation, and regulate the response to antigen. In general, cytokines are paracrine rather than endocrine mediators. Therefore, many cytokines possess a relatively short half-life following administration; consequently, repeated bolus injections are necessary to achieve pharmacologically relevant levels. This may result in a reversal of the natural immune response: a high level of cytokine in circulation and a low level trafficking to the tumor site or involved lymphoid organs. These levels of systemic cytokine are often accompanied by mild to severe side effects, such as hypotension, cachexia, fever, chills, vomiting, and diarrhea. Since cytokines primarily exert their effects locally, expression of cytokine by tumor cells would ensure that cytokine would be available during T cell interaction with the tumor cell, presumably enhancing recognition of tumor antigens and/or increasing T cell proliferation. Expression of cytokine by tumor cells also avoids the side effects of systemically administered cytokine while achieving effective concentrations locally. Tumor cells transduced with cytokine genes, even when irradiated, can secrete cytokine for several days and provide sustained delivery.

A multitude of cytokines have been transduced into tumor cells to construct tumor vaccines. Several excellent reviews of cytokine gene-transduced tumor vaccines are recommended for comprehensive analysis of the animal studies performed using this approach [17,109–111]. In most cases, expression of cytokine above a threshold level abrogated tumor formation. However, rejection of the cytokine expressing tumor did not necessarily result in immunity against the parental cell line. In some cases, especially in tumors transfected with interferon genes, expression of cytokine inhibited growth of the tumor and sometimes contributed to rejection without inducing protective immunity. Most cytokines also provoked a local inflammatory response which was sufficient to eliminate the tumor inoculum but did not protect against challenge with parental tumor cells. Some cytokines appeared to be effective only in some tumor models, but not others. However, particular cytokines in select tumor models were effective in inducing immunity to nontransduced parental tumor cells. One study, which compared several cytokines in a single model, utilized the nonimmunogenic B16-F10 murine melanoma [112]. The B16 cells were transfected with IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, IL-1Rα, ICAM, CD2, or TNF-α, then tested for their ability to stimulate immunity to the parental tumor. Only GM-CSF-transduced tumor cells were able to protect against challenge with parental tumor cells. In addition, injection of irradiated GM-CSF-expressing tumor cells prolonged survival of mice which had established B16 tumors. More recent studies in the same model suggest that the antitumor immunity activated by GM-CSF-expressing B16 cells could modestly extend the survival of mice with B16 tumors in the brain [113]. GM-CSF has also been shown to be effective in models of prostate and bladder cancer [114,115]. In addition to the numerous trials utilizing systemic GM-CSF, a phase I/II study of in vivo transfer of the GM-CSF gene into melanoma lesions is underway.

IL-2-transduced tumor cells were shown to stimulate rejection of established tumors of bladder, melanoma, pancreas, and breast in animal models [116–119]. IL-2 has been widely used in a variety of cancers with demonstrated effectiveness in melanoma and renal carcinoma. Because of the side effects associated with systemic IL-2, use of IL-2 gene-modified tumor vaccines offers a method of delivering effective levels of IL-2 with lower toxicity and mortality rates.

More recent studies made use of IL-12 gene-modified tumor cell vaccines. IL-12 was originally defined as natural killer stimulatory factor (NKSF), and is active against a variety of tumors when given systemically. IL-12-expressing fibroblasts were able to slow the growth of established melanoma, and IL-12-secreting fibrosarcomas also suppressed growth of parental tumor cells [120,121]. A recent comparison of IL-12- and IL-2-transduced colon carcinoma cells found that IL-12-secreting tumor cell vaccines were much more effective in treatment of lung metastasis [122]. Animals given IL-12-producing vaccines accumulated more lymphoid cells in the lung and demonstrated a 40% rate of cure. This study was particularly relevant because the investigators used C26, a syngeneic tumor cell line of the same histologic type, to treat C51 metastases. This more closely resembled the use of allogeneic vaccines as used in several clinical trials.

Transduction of costimulatory molecule genes offers another approach to enhance tumor immunogenicity.

Among the most important costimulatory molecules are CD80 (B7-1) and CD86 (B7-2) [123,124]. The interaction of the CD28 receptor on T cells with the CD80 or CD86 molecules on antigen-presenting cells is crucial for T cell activation [125–127]. If T cells encounter antigen but do not receive a signal through the CD28 receptor, they are functionally crippled or ''anergic.'' Since expression of CD80 and CD86 is limited to B cells, macrophages, and dendritic cells, tumor cells are capable of inducing T cell anergy rather than stimulation and activation.

Initial reports demonstrated that B7+ tumor cells were rejected and, in some cases, this resulted in immunity to the parental tumor cells [128,129]. Further studies indicated that the effectiveness of B7 transfected tumor cells was related to the immunogenicity of the parental tumor [130]. Tumors which were already immunogenic demonstrated enhanced immunogenicity following transfection of the B7 gene, but the effect on nonimmunogenic tumors was minimal. Furthermore, experiments by Huang et al., among others, raised doubts about the mechanism by which B7 enhances immunogenicity [131]. By utilizing bone marrow chimeras, Huang and associates were able to show that bone marrow-derived antigen-presenting cells were responsible for presenting tumor antigens to T cells in vivo. B7-expressing tumor cells could also stimulate tumor-specific T cells (but much less efficiently) [132]. Other studies showed that expression of B7 renders target cells more susceptible to lysis by NK cells [133], which may be the major contribution of B7 to immunogenicity of tumor cells. Enhanced lysis of B7+ tumor cells would release tumor antigens, making them more available for presentation by bone marrow-derived antigen-presenting cells. A clinical trial of a B7-transfected allogeneic melanoma vaccine is underway.

A number of groups attempted to enhance tumor cell immunogenicity by their transfection with alloantigens. Since alloantigens are recognized by a high percentage of T cells, tumors expressing alloantigens should evoke a vigorous T cell response. The hope is that the production of cytokines in response to alloantigen would provide a favorable environment for the recognition of tumor antigens. Using this approach, two different clinical trials were established utilizing injections of the gene for HLA-B7 (not to be confused with the costimulatory molecule, B7) directly into lesions of metastatic melanoma [134,135]. Frequent toxicity, associated with technical aspects of the procedures, was noted—along with pain, hemorrhage, pneumothorax, and hypotension. However, the in vitro and in vivo parameters assessed were encouraging. DNA, RNA, and protein for HLA-B7 were detected in most treated lesions. In a single patient from the first study, regression of both treated and distal lesions was detected [134]. In the second report, seven patients (50%) had tumor responses, defined as a >25% decrease in the size of the injected nodule by radiologic or physical examination [135]. Of great importance, lesions distal from the injection site were also affected. Studies in renal and colon carcinoma utilizing this approach were disappointing, with no responses to therapy observed [136,137].

Another consideration which is relevant to clinical application is the mode of gene transduction. The relative merits of viral vectors and the methods of transfecting plasmid DNA are beyond the scope of this review. However, in order to evaluate experimental data, the barriers to application of modified tumor cell vaccines must be considered. One potentially substantial barrier is the availability of autologous tumor cells for gene transfer to a sufficient population of potential patients. Individualized immunotherapy overcomes some of the potential limitations of the process (such as the frequent requirement of haplotype matching), but isolating and cell culture expansion of tumor from every patient can be extremely labor intensive. Moreover, not all types of neoplasms (e.g., prostate) are amenable to sufficient harvests of tumor tissue. This leads to two of the more common alternative approaches: the use of allogeneic tumor cells, or in vivo gene transduction. Allogeneic tumor cells need not share MHC alleles with the intended recipient to be effective; they merely need to express shared TAA. Both experimental and clinical studies made use of allogeneic tumors as vaccines. The feasibility of allogeneic tumor cell vaccines will be clarified as more TAA are characterized. If TAA common to tumors of a given histologic type can be identified, then allogeneic tumor vaccines will be more widely applicable. The melanocyte differentiation antigens, tyrosinase, gp100, gp75, and MART, are examples of antigens expressed by most melanomas—a finding which supports the use of allogeneic melanoma cell lines as vaccines. The use of allogeneic cells offers the additional benefit of stimulating strong immune responses to the allogeneic MHC antigens.

In vivo gene transfer proposes to introduce DNA directly into a tumor mass to generate cytokine-producing tumor cells. This has been accomplished using DNA in liposomes, naked DNA, and viral vectors injected into the tumor. Expression of the plasmid or viral DNA has been demonstrated using each of these methods. There are two major shortcomings to this strategy: (1) the need for access to the tumor, which requires surgery and its possible complications, and (2) in most cases, only those tumor nodules at, or proximal to, the inoculation site are affected. This suggests that not only would each metastasis need to be injected with DNA, but that the mechanism of tumor regression is the local inflammatory response, not induction of specific immunity. Ideally, the destruction of one lesion would provide sensitization to shared tumor antigens, triggering rejection of distant me-

tastases. Unless this outcome can be achieved, in vivo gene transfer may remain of limited use.

GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR (GM-CSF)

GM-CSF is a glycoprotein that helps regulate the maturation, production, and function of the granulocyte and monocyte-macrophage subpopulations of human white blood cells [138,139]. GM-CSF has pronounced effects on the immune system, such as: (1) upregulating MHC class II expression, (2) modulating other hematopoietic growth factors, (3) eliciting localized inflammatory responses at the site of antigen deposition, (4) stimulating myeloid precursors in bone marrow, and (5) promoting dendritic cell migration and development [140–143]. As far as the effect on DC is concerned, GM-CSF potentiates several in vitro effects that would suggest its being advantageous as an adjuvant in immunotherapeutics. The addition of GM-CSF to peripheral blood DC has been shown to prolong cell viability beyond 30 days of culture. Such long-lived DC remain immunostimulatory, as evidenced by their ability to provoke T cell proliferation in both autologous and allogeneic mixed leukocyte reactions (MLR). However, total cell number does not increase over time, implying the inability of GM-CSF to induce DC division and proliferation [144].

INTERLEUKIN-12 (IL-12)

Clinical immunotherapeutic trials in humans using IL-12 as adjuvant or single reagent have recently been initiated. Increasing numbers of phase I clinical trials are being planned with IL-12, and because there is a scarcity of published human studies, we will present instead some relevant preclinical studies.

IL-12, a heterodimeric cytokine originally shown to be secreted by phagocytic cells as part of the innate immune repertoire, has more recently demonstrated effective antitumor activity. It is produced by a wide range of cells, including macrophages, monocytes, B cells, granulocytes, keratinocytes, mast cells, and DC. It promotes the development and growth of preactivated T and NK cells, and augments T- and NK-mediated cytolytic activity. IL-12 stimulates T and NK cells to produce several cytokines, such as IFN-γ, TNF-α, IL-2, IL-3, IL-8, IL-10, and numerous colony-stimulating factors. It also directs the naive T_H cell towards a T_H1 (effector cell) phenotype while concurrently promoting its expansion (reviewed in [145–147]. IL-12 does not exert its antitumor effects directly [120,148], most likely because only T and NK cells express the cognate receptor.

Brunda et al. [149] administered systemic IL-12 five times per week for a total of 4 weeks to mice previously injected with B16F10 melanoma cells. Growth of tumor from various histologic origins was significantly decreased across several treated mouse strains as compared to control animals. In fact, the therapeutic value of IL-12 was noted even when treatment did not commence until day 28 after tumor cell introduction, when tumors were quite established. Based on results with various strains, such as the NK cell-deficient *bg/bg* beige mouse, the antitumor effect was apparently mediated largely by $CD8^+$ cells, and not by $CD4^+$ or NK cells [149]. IL-12 is also highly effective when used to gene-modify effector cells. Tahara and coworkers [120] transfected the two cDNAs encoding the p35 and p40 chain of IL-12 under the control of a SV40 promoter into the murine fibroblast line, NIH-3T3. Admixing of IL-12-transfected fibroblasts with a weakly immunogenic, B16-derived murine melanoma line was shown to be of great therapeutic benefit by significantly delaying the onset of tumor growth. Immunohistochemistry revealed that tumor cells injected with gene-modified effector cells resulted in fibroblast encapsulation, but increased $CD4^+$ T cell infiltration was not observed [120]. Local secretion of IL-12 mediated its effects in a dose-dependent manner, as low titers of IL-12 (1.2 units/5×10^5 cells/48 hours) provided maximal inhibition of established tumor, while higher titers were not advantageous. To enhance the potency of its antitumor effect, IL-12 was later given in combination with IL-2 and IFN-α. In murine renal cancer, combination therapy with IL-2 plus IL-12 resulted in 90–100% of treated animals remaining tumor-free, as compared with 10–30% of animals given IL-12 alone. However, when administered with doses of IL-2 or IFN-α that were maximal in a single-agent approach, toxicity and mortality rates rose substantially. Treatment with IL-12 plus suboptimal amounts of IL-2 or IFN-α, while reducing substantially the toxicity and death rates, also severely mitigated the antitumor effects [146]. Thus, a balance between therapeutic benefit and side effects still needs to be achieved.

Research involved with antiangiogenic factors capable of cutting off a tumor's blood supply, either by preventing new vessel formation or by blocking those already formed, suggests an additional role for IL-12 in tumor inhibition. Using both normal and various immunocompromised mouse strains, Voest et al. [150] examined the effect of IL-12 in a model of basic fibroblast-growth factor-induced corneal neovascularization. In normal, T cell-deficient, and T and B cell-deficient mice, IL-12 administration largely suppressed vessel formation. This effect was reversed by concomitant administration of anti-interferonγ (INFγ) MAbs, suggesting that antiangiogenesis was mediated through this cytokine [150]. However, in a earlier report, injection of anti-NK-cell MAb, but not anti-INF-γ MAb, was able to block the enhancement of INF-γ-producing T cells [151]. These findings imply that IL-12 may promote an antitumor effect via more than one pathway, including one distinct from that mediated via various immune effector cells. Following the observation that tumors in IL-12-treated mice dis-

played a severely diminished system of blood vessels, it was established that IL-12 promoted production of an antiangiogenic protein, IPO-10, and is currently the basis of an IL-12-based phase 1 clinical trial for the treatment of kidney cancer [152].

INTERFERON-α (IFN-α)

IFN has proven to be an effective agent in the treatment of melanoma and renal carcinoma. In renal carcinoma, responses are seen in 20% of patients treated with subcutaneous injections of IFN-α, and similar response rates are seen in patients with melanoma. The proposed mechanisms of action include upregulation of MHC and tumor-specific antigens and inhibition of tumor cell proliferation. Since IFN-α may be important in priming tumor-specific lymphocytes—and IL-2 would act to expand those lymphocytes—treatment with both IFN-α and IL-2 might yield a complementary pairing of effects. Evidence for this has been observed in clinical trials of IFN-α and IL-2 in metastatic renal carcinoma in which responses were seen in 30% of patients. Combined IFN-α and IL-2 did not seem to significantly benefit patients with metastatic colorectal carcinoma, but this might reflect the lack of any effectiveness of either cytokine in this tumor type [80]. IFN-α has also been paired with other immunotherapy protocols. In one such study, IFN-α was given to renal carcinoma patients prior to nephrectomy. Tumor infiltrating lymphocytes were isolated from the tumor, cultivated in IL-2 and infused back into the patient. Continuous infusion of low-dose IL-2 began, following injection of TIL. The overall response rate of 30% achieved in this regimen was greater than that attained with IFN-α or TIL alone. In addition, the responses appeared to be more durable than those observed with IFN-α and IL-2 combination therapy [98]. IFN-α has also been given in melanoma patients who had failed previous treatment with an allogeneic melanoma vaccine [153]. Forty percent of patients responded as measured by regression of tumor. While this response rate is greater than the response to IFN-α or vaccine alone, the study design did not allow comparison of combination therapy with either single therapy. In 1995, Doveil and coworkers reported that IFN-α-2b treatment (3 MU i.m. three times a week) was well tolerated and increased 5-year survival from 36% to 62% in patients with stage IIIb melanoma [154]. However, alternative therapies were not tested concurrently.

A recent report from the Eastern Cooperative Oncology Group (ECOG) [155] also demonstrated the potential clinical benefit of IFN-α-2b therapy in the treatment of melanoma. Two hundred fifty-two patients were treated with maximum-tolerated doses of 20 MU/m^2/d intravenously for 1 month and 10 MU/m^2 three times per week subcutaneously for 48 weeks. Using one-sided statistical analysis (*P*-test), a significant enhancement of relapse-free survival was obtained following periodic examination of tumor burden and the presence or absence of regional lymph node metastasis. Five-year survival rates were improved from 37% to 46%. However, severe toxicity was noted: 67% of all patients demonstrated grade 3 toxicity, and two patients succumbed to lethal hepatic toxicity [155]. Using a different method of statistical analysis (two-sided *P*-test) and quality-of-life survival analysis (Q-TWiST), a reinvestigation of the same patients concluded that the improvement was significant only for those patients who considered toxicity to have a high relative value and relapse to have a low relative value [156]. Thus, although the clinical efficacy of high-dose IFN-α-2b could counterbalance contraindications, whether or not this is a preferred treatment might depend on the patient's tumor burden and attitudes concerning quality of life. Lastly, some clinicians have stressed the importance of using appropriate types of statistical analysis before drawing conclusions. For example, it may be more appropriate to use a two-sided instead of a one-sided *P*-test (used in the ECOG report), the latter of which may be more suitable when there is an a priori presumption that a treatment could only be advantageous [157].

The literature suggests that the potential clinical benefit of IFN-α in the treatment of cancer is dependent upon cancer type. When seven randomized trials for Chronic Myeloid Leukemia (CML) were analyzed, it was evident that regimens utilizing IFN-α produced a significant improvement of 5-year overall survival rates (from 42% to 57%), as compared with standard chemotherapy treatments with drugs such as busulfan or hydroxyurea [158]. The improvement was independent of sex, age, or risk group, although the number of patients studied who did not have the Philadelphia chromosome abnormality were too small for analysis. However, for patients with advanced colorectal cancer, the addition of IFN-α-2b to standard therapies has not proved helpful. When used as an adjuvant to single modulation chemotherapy (leucovorin [folinic acid] concurrently with fluorouracil [5-FU]), IFN-α-2b (5 MU s.c. three times weekly) actually decreased overall survival rates and had more frequent toxicity and contraindications (including neutropenia, anemia, diarrhea, flu-like syndrome) as compared with the group given only leucovorin and 5-FU [159]. In a second report, the combination of IFN-α-2b with 5-FU did not provide any benefit over treatment solely with 5-FU [160]. In summary, when used alone or in combination with other immune-based procedures, the aforementioned studies suggest that IFN-α has potential therapeutic benefit for certain cancer types—the extent of which awaits long-term follow-up.

CONCLUSIONS

Immunotherapy is an approach to cancer treatment still in its relative infancy. For many of the protocols or potential protocols described, the first comprehensive clinical trials are currently underway or in the planning stages. The coming years should produce an abundance of data as to the efficacy of the many and varied antitumor methodologies used. Standard clinical parameters to determine therapeutic benefit, such as tumor burden, should be combined with the in vitro assessment of immune reactivity to treatment, including—at minimum—skin testing (delayed-type hypersensitivity) and cytokine secretion profiles of patient immune cells. At present, each experimental therapy charts its own course for treatment; e.g., how often to treat and at what dosage, which makes the prospect of standardized immunotherapy seem a distant hope. Thus, it is crucial that as many parameters be measured as is feasible to enable conclusions to be drawn about how best to treat cancer patients using immunotherapy. Mere reporting of toxicity and the percentage of responders will not contribute sufficient information toward this goal. Regardless of the regimen, it is hoped that the clinician and bench scientist will join forces in comprehensive patient monitoring. With so many studies underway, the potential amount of information is great. Putting such extensive information to use, we can more quickly optimize the efficacy of treatment, to the ultimate benefit of all those involved.

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